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Assignment of two ultrastructures formed by a mixture of hexonamides using autoradiography and electron microscopy

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SUMMARY

The combined application of autoradiography and electron microscopy allowed the assignment of molecular components to individual micellar fibres in a mixed gel. Resolution was of the order of 0·1 μm. As a result, it was shown that bimolecular sheets of *N*-dodecyl-L-mannonamide (= L-Man-12) completely separated from helical rods consisting of tritiated *N*-octyl-D-gluconamide (= D-Glu-8). The method should be useful for the analysis of several other synthetic supramolecular systems.

INTRODUCTION

The aggregation behaviour of *N*-alkyl-hexonamides and subsequent formation of the characteristic ordered structures has recently been described (Pfannemueller & Welte, 1985; Fuhrhop *et al.*, 1988). Such structures were formed by cooling hot micellar aqueous solutions down to temperatures where amide hydrogen bond chains are formed in a co-operative reaction sequence. The D- and L-forms of *N*-octyl-gluconamide and *N*-dodecyl-gluconamide (D, L-Glu-8, 12) aggregate to form ultrathin helices with diameters of around 7 and 9 nm respectively (Fuhrhop *et al.*, 1987), which continue to rearrange to achieve thicker fibres (Fig. 1a). The D- and L-forms of *N*-octyl-mannonamide (D, L-Man-8) form 'cigar-like', rolled-up sheets (Fig. 1b), whereas the D- and L-forms of *N*-octyl-galactonamide (D, L-Gal-8) appear as twisted ribbons (Fig. 1c). The 'screw sense' of the chiral aggregates is specific and correlates with the configuration of the sugar head group (Fuhrhop *et al.*, 1988).

In view of this ordered aggregation behaviour characteristic of the individual compounds, we investigated whether the micellar solutions of hexonamides differing in: (i) the sugar head-group stereochemistry and (ii) the length of alkyl chain upon mixing and subsequent cooling, do produce a new form of ordered ultrastructures or remain separated. In a systematic study of gluconamides, mannonamides and galactonamides mixtures, cases of fibre separation, as well as of 'alloy' formation (depending on the

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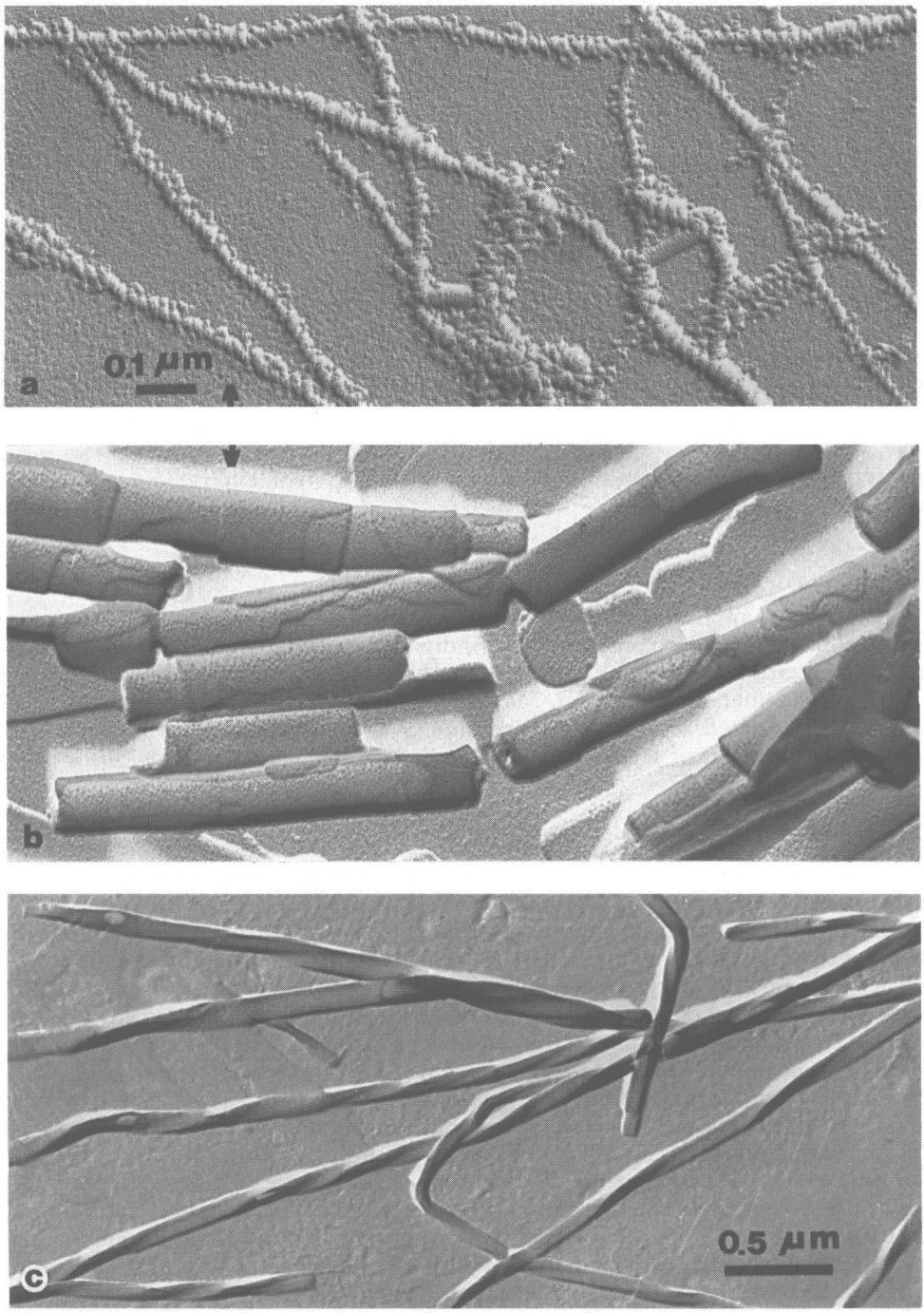

Fig. 1. Electron micrographs of ultrastructures as formed by individual hexonamides. Pt/C shadowed. (a) Multiple helices of the *N*-octyl-D-gluconamide (D-Glu-8). (b) 'Cigar-like' rolled-up sheets of the *N*-octyl-D-mannonamide (D-Man-8). (c) Twisted ribbons of the *N*-octyl-L-galactonamide (L-Gal-8).

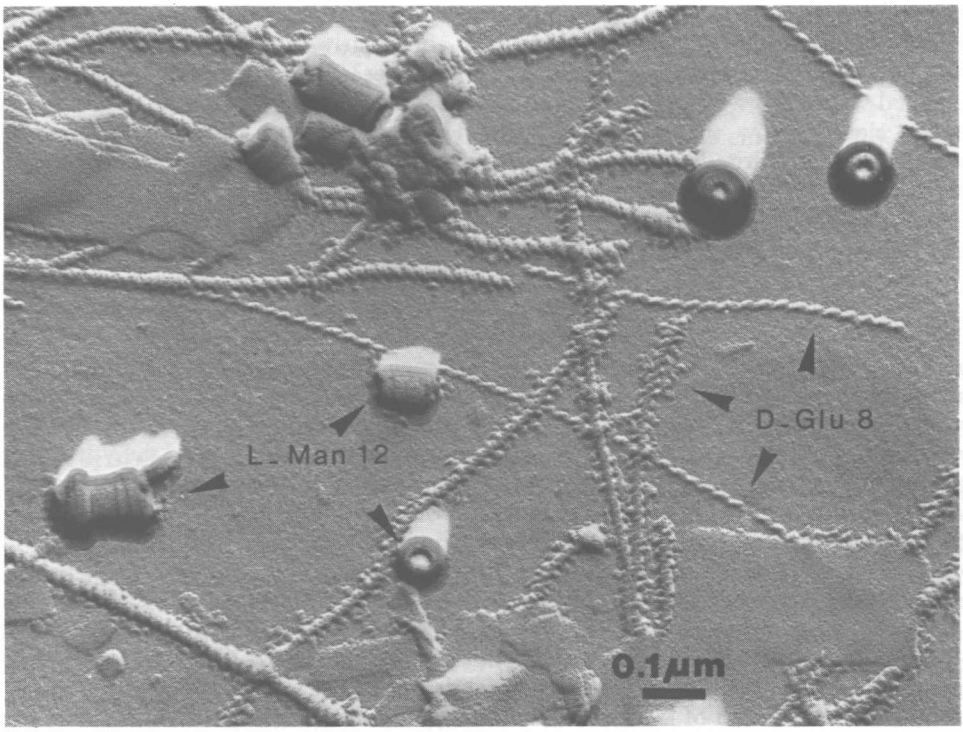

Fig. 2. Electron micrographs representing ultrastructures formed by an equimolar mixture of *N*-octyl-D-gluconamide (D-Glu-8) and *N*-dodecyl-L-mannonamide (L-Man-12). Pt/C shadowed. The sample was taken during stage 2 of gel formation at the first appearance of turbidity. Note the left- and right-handed helical structures formed by D-Glu-8 as well as small, rolled-up sheets of L-Man-12.

mixed stereoisomers) were observed (Fuhrhop & Boettcher, 1990). Of particular interest were the systems where different stereoisomers formed two separated micellar fibres. It was assumed that one of the fibres could then be considered to act as an electron donor (e.g. with benzidine) and the other as an electron acceptor (e.g. with a quinone). The excited states of an added photosensitive dye may induce an electron transfer from fibre A to fibre B. In this way, it should be possible to obtain a light-induced charge separation after the reduction of the oxidized dye by fibre A. Such a 'de-mixed' state of the components was observed by electron microscopy, in the case of an equimolar mixture of D-Glu-8 and L-Man-12 (see Appendix) where we obtained both multihelical fibres—as already known from the aggregation behaviour of the D-Glu-8—as well as 'cigar-like' cylinders typical of the D-Man-8 and L-Man-8 (Fig. 2). In order to provide evidence of the quantitative separation and the fact that each structure is formed by one individual compound, an autoradiographical technique was used and one of the mixture components, namely D-Glu-8, was radiolabelled. Although the task appears simple, no direct chemical or spectroscopic method other than autoradiography was available to determine the purity of the individual aggregates. Spectroscopic methods are, of course, widely used for determining phase separation, but are not able to localize the various structures with the required resolution of about 100 nm. This is the first description of the assortment of soft lipid structures in mixed systems at high significance level.

There were several uncertainties at the start of this work. Many questions were open; such as would the lipid aggregates fuse with the photographic layers during the

long exposure time required, or would they spread? Would slow, co-crystallization occur? Would resolution and sensitivity be sufficiently high in order to allow the assignment of the radiolabel to specific micellar fibres? The only positive results so far achieved were with well-defined biological structures or polymers. The experimental results clearly show that synthetic supramolecular systems can also be analysed successfully by autoradiography of easily accessible tritiated samples and electron microscopy.

MATERIALS AND METHODS

Synthesis of gluconamides

D-[6-^{3}H]-glucose, with a specific activity of 500 mCi/mmol (Amersham), and 178·2 mg unlabelled D-glucose in 10 ml triple-distilled water, were electrolytically oxidized for 16·08 min at 200 mA in the presence of calcium bromide and calcium carbonate (Frush & Isbell, 1963; Emmerling & Pfannemueller, 1978). Excess bromide was precipitated with silver carbonate in dark-room conditions. The filtered solution was then treated with a strongly acidic ion-exchange resin (Merck) and dehydrated by azeotropic distillation with 1-butanol. The obtained D-[6-^{3}H]-glucono-γ,δ-lactone was treated with *n*-octylamine in methanol at boiling point for 1 h. *N*-octyl-[6-^{3}H]-gluconamide was recrystallized twice from methanol/water and solid-washed with water. The yield was 50% (= 155 mg), the melting point 158° C, the R_f = 0·75 (ethanol, silanized silica gel) and $[\alpha]_{25}^{D}$ = + 28·3° (in DMSO). Unlabelled D-Glu-8 and L-Man-12 were obtained from the aldono-δ-lactones (Sigma) by aminolysis with *n*-octylamine or *n*-dodecylamine in methanol. The yield of D-Glu-8 was 90%, the melting point 158°C, the R_f = 0·75 (ethanol, silanized silica gel) and the $[\alpha]_{25}^{D}$ = + 28·3° (in DMSO). The yield of L-Man-12 was 63%, the melting point was 158–159°C and $[\alpha]_{50}^{D}$ = + 17·3° (in DMSO).

The spectra (IR, ^{1}H-NMR and MS) and elemental analysis (C, H, N) of D-Glu-8 were in agreement with published values (Fuhrhop *et al.*, 1987). L-Man-12 fitted very well with expected spectroscopical data.

Electron microscopy of gels

The equimolar mixture of D-Glu-8 and L-Man-12 was heated in double-distilled water to 100°C, and a clear, micellar solution was obtained. On cooling, the solution became turbid (80°C) and a solid white gel was formed at *c.* 40°C. The gel thus obtained was labile and decayed after a few minutes. The process of structure formation while cooling was followed by time-resolved preparation for the electron microscope. Formvar-coated copper grids (400 mesh, Balzers) were dipped into the solution of four macroscopically discernible stages: stage 1 – clear micellar solution; stage 2 – first appearance of turbidity; stage 3 – compact gel (approximately 40°C); stage 4 – gel at room temperature prepared 30 min after stage 1. The grids were covered on one side with Parafilm to avoid a double-sided loading of gel material. Excess material was blotted off with filter paper and air-dried. The specimens were 'shadowed' by evaporation with platinum/carbon at an angle of 35° using the Edwards Coating System E 306 A. Electron microscopy was carried out on a Philips EM 300 using an accelerating voltage of 80 kV at principal magnifications of between 5000 and 27,000.

Autoradiography

For our experiments, we used Formvar-coated gold grids (400 mesh, Balzers) which were covered on one side with Parafilm (again, to avoid loading of the material on both sides); on the Formvar side, a 5-nm layer of carbon at an angle of 90° was coated. The preparation followed the analogous conditions of unlabelled material by dipping the

grids in the equimolar mixture of *N*-octyl-D-[6-^{3}H]-gluconamide and *N*-dodecyl-L-mannonamide during the gel formation (stage 3).

The specimens were 'shadowed' with platinum/carbon by evaporation at an angle of 35° and to avoid chemography (Salpeter & Bachmann, 1972; Williams, 1972), the grids were then covered with a 5-nm carbon layer (90°). The emulsion (L4, Ilford) was drawn up using the platinum-loop method of Haase & Jung (1964) under safe light conditions (Ilford's Safe light no. 902, light brown). The emulsion (10 g) was diluted with 8·2 ml double-distilled water and 0·8 ml 0·2% solution of manoxol (dioctyl-sodium-sulphosuccinate, Sigma) (Rogers, 1967) was added. The thickness of the emulsion layer was determined by interference colours under safe light using the copper-shining part of the film, which is approximately 120 nm in width (Maraldi *et al.*, 1972).

The exposure was carried out at 4°C in light-proof boxes for a period ranging from 64 to 120 days, because it was calculated that under these conditions, about 1·5 grains/μm^2 could be expected after an exposure period of 60 days. However, the thickness of the sample was never uniform, causing variations in radioactive decay on the grid surface and making it difficult to obtain a precise calculation of the optimum period for exposure.

In the development of autoradiographs, two general methods were followed. We used the Gold-Latensification-Elon-Ascorbinic Acid (GEA) developer, according to Wisse & Tates (1968) and the Phenidon-developer, following the method of Lettre & Paweletz (1966). The fixation of developed autoradiographs was accomplished with a 25% solution of sodium thiosulphate. The developed grids were dried overnight and electron microscopy was carried out using the Philips EM 300 at 80 kV with principal magnifications of between 5600 and 12,500.

RESULTS AND DISCUSSION

The ultrastructures formed in the mixed systems of various hexonamide mixtures were systematically examined. One result was that 1,3-syn interactions in the gluconamide head group (see Horton *et al.*, 1983) induce the curvature of the micelleous rods. In the mannonamide, such steric hindrance does not occur and less defined structures such as uniform sheets or platelets are formed (Fuhrhop & Boettcher, 1990). *N*-dodecyl-L-mannonamide (L-Man-12) was insoluble in refluxing water, but formed clear, micellar solutions when mixed with equal amounts of *N*-octyl-D-gluconamide (D-Glu-8). On cooling the D-Glu-8 plus L-Man-12 system to room temperature, the simultaneous appearance of multihelical fibres and 'cigar-like' cylinders was observed immediately (Fig. 2). These fibres were identical both in shape and size to the respective structures formed by the individual components alone. Surprisingly, however, gluconamide fibres having both left-handed and right-handed helices were formed (Fig. 2) in the mixed system. It is assumed that a statistical screw dislocation occurred during the fast growth of glucon fibres from mixed micelles.

Upon further ripening of the gel, the size and multiplicity of the structures increased in several typical forms as shown in Fig. 3(a). After about 5 min, the disintegration of the gel led to the appearance of isotropic platelets (Fig. 3b). To provide direct evidence that there is actually a quantitative separation of the components despite their being in one solution, we applied the autoradiography technique. Tritium-labelled D-Glu-8 and unlabelled L-Man-12 were prepared in conditions identical to those with the non-radioactive mixture. On the electron micrographs from the autoradiographic experiments, four different structures could be observed: helical fibres, cylinders, isotropic and anisotropic platelets (Fig. 4). The distribution of the grains over the different structures was quantitatively examined. The significance of the difference in the distributions was determined using the χ^2 method (Sachs, 1968). The evaluation of

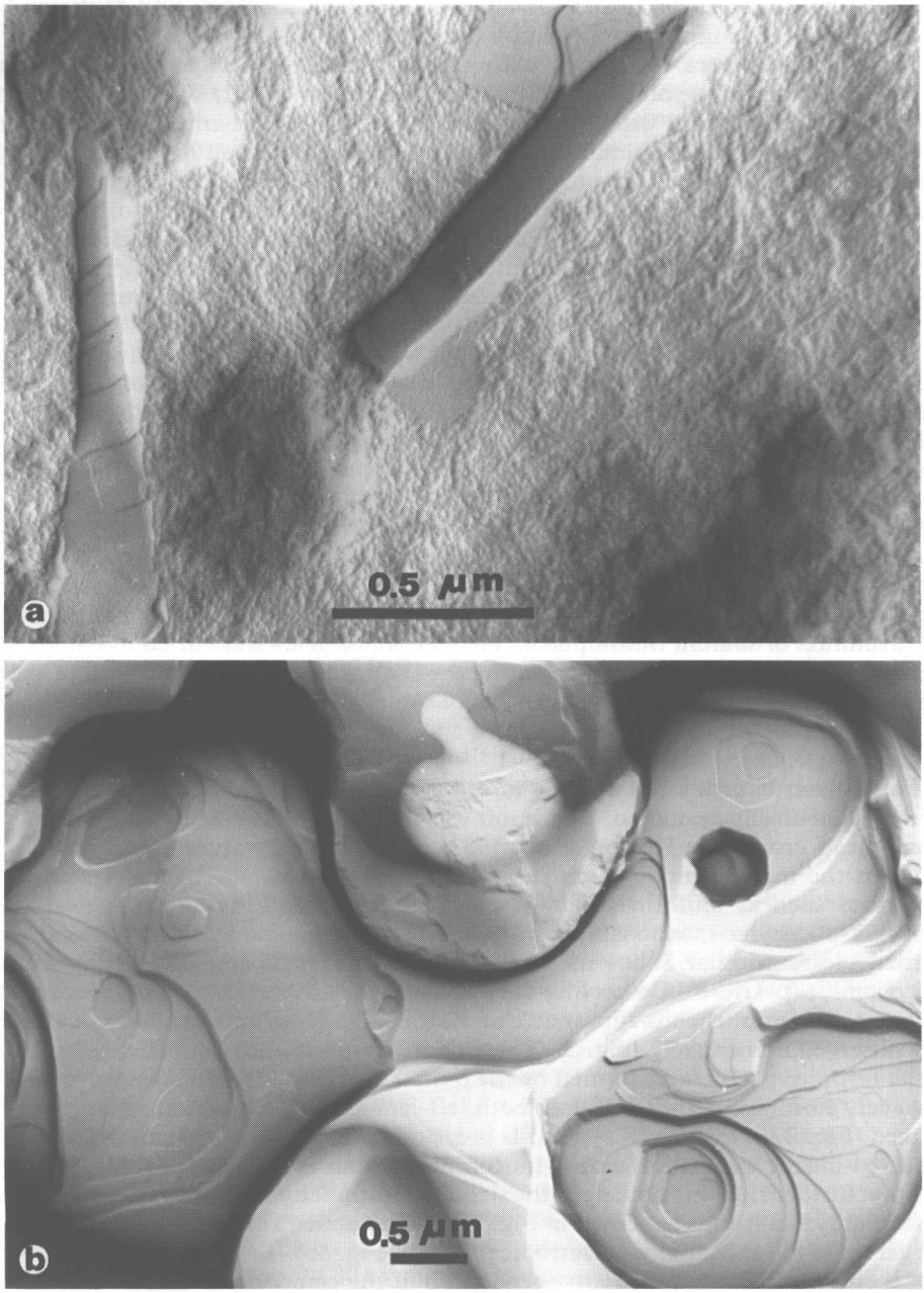

Fig. 3. Electron micrographs showing continued growth of ultrastructures formed by the mixture of D-Glu-8 and L-Man-12 in later stages. (a) Stage 3, gel; (b) stage 4, cold gel (room temperature).

representative parts of the best ten micrographs (by counting several thousand grains) provided the following information at a significance level of 0·1%: helical fibres (Fig. 4a, c, d) and isotropic platelets (Fig. 4b) are radioactive and contain D-[6-^{3}H]-Glu-8. Anisotropic sheets (Fig. 4a) and cylinders (Fig. 4b, c, d) do not contain labelled material.

It is concluded that clearly defined sheets and scrolls contain mannonamide with less than 0·1% of gluconamide admixture. The rapid decay of the aqueous gel was accompanied by the appearance and growth of their crystallites with rounded edges. These platelets contain labelled gluconamide material (Figs. 3b and 4b). In order to check for artefacts in the preparation, the procedure was repeated several times and the same type and proportions of superstructures was always found. No significant differences were found between frozen-hydrated, freeze-dried and freeze-etched specimens.

Occasionally, silver grains were found just above the 'mannon structures', which could be attributed to (i) overlying fibres of D-Glu-8 or (ii) the background of the preparation. For the determination of the background, unlabelled mixtures with L4-emulsion were prepared under identical conditions. It is well known that the background variation depends on the type of developer used. For GEA-developer, 1 grain per 150 μm^2 was determined. Fischer & Werner (1971) report 1 grain per 30 μm^2. In the case of the Phenidon developer, 10 ± 3 grains per 1000 μm^2 were determined (Gupta *et al.*, 1973), whereas we could not detect any grain for the unlabelled mixture. Additional account should also be taken of the average amount of 2–3 grains per AgBr crystal hit (Fakan & Fakan, 1987). The choice of developer was determined by the size and shape of the grains. More common developers such as Kodak D-19 show fibre-like grains and give no indication of the origin of the AgBr crystal which was hit. We used both developers, because of their differing efficiencies and backgrounds, and reached the same qualitative conclusions from each. One of the primary problems in autoradiography is the loss of resolution caused by the lateral spreading of the image (Williams, 1985). Starting from a certain distribution of developed grains, it is necessary to identify accurately the source of radioactivity over the various structures. A practical method of resolution is given by the radius of a circle around a source which encloses 50% of the developed grains; this criterion is defined as HD value. The influence on resolution from a varying number of factors is extensively discussed and summarized by Gupta *et al.* (1973) and Delain & Bouteille (1980). On the basis of their reports, the resolution for a tritium-labelled source combined with the use of Ilford's L4 emulsion gives an HD value of about 160 nm, depending on the developer used. Here the resolution (in the case of Phenidon developer) was determined by following the method of Delain & Bouteille (1980). The HD value between the isolated gluconamide fibres and the nearest developed grains was found to be 60 nm. This is less than reported by Delain & Bouteille (1980). However, because our objective was to assess the relative distribution of silver grains over the structures formed by two different compounds, the significance was tested by the χ^2 method and it was noted that the source of silver grains was the gluconamide with a confidence of 99·9%.

The possibility of grains also occurring due to contamination from free ^{3}H-glucose (not associated with the ultrastructure) can be ruled out because the labelled gluconamide was recrystallized twice from ethanol/water and the crystals washed with double-distilled water to remove any soluble glucose.

In conclusion, tritium-labelled gluconamide molecules mixed with another hexonamide could be located satisfactorily by autoradiography. The relatively long period between the formation of gel and the detection by autoradiography was not a limiting factor in our experiments because a slow spreading or crystallization of the lipid molecules could have occurred during the time of storage. The method introduced in this paper should be of general interest for structural studies of mixed, synthetic supramolecular systems.

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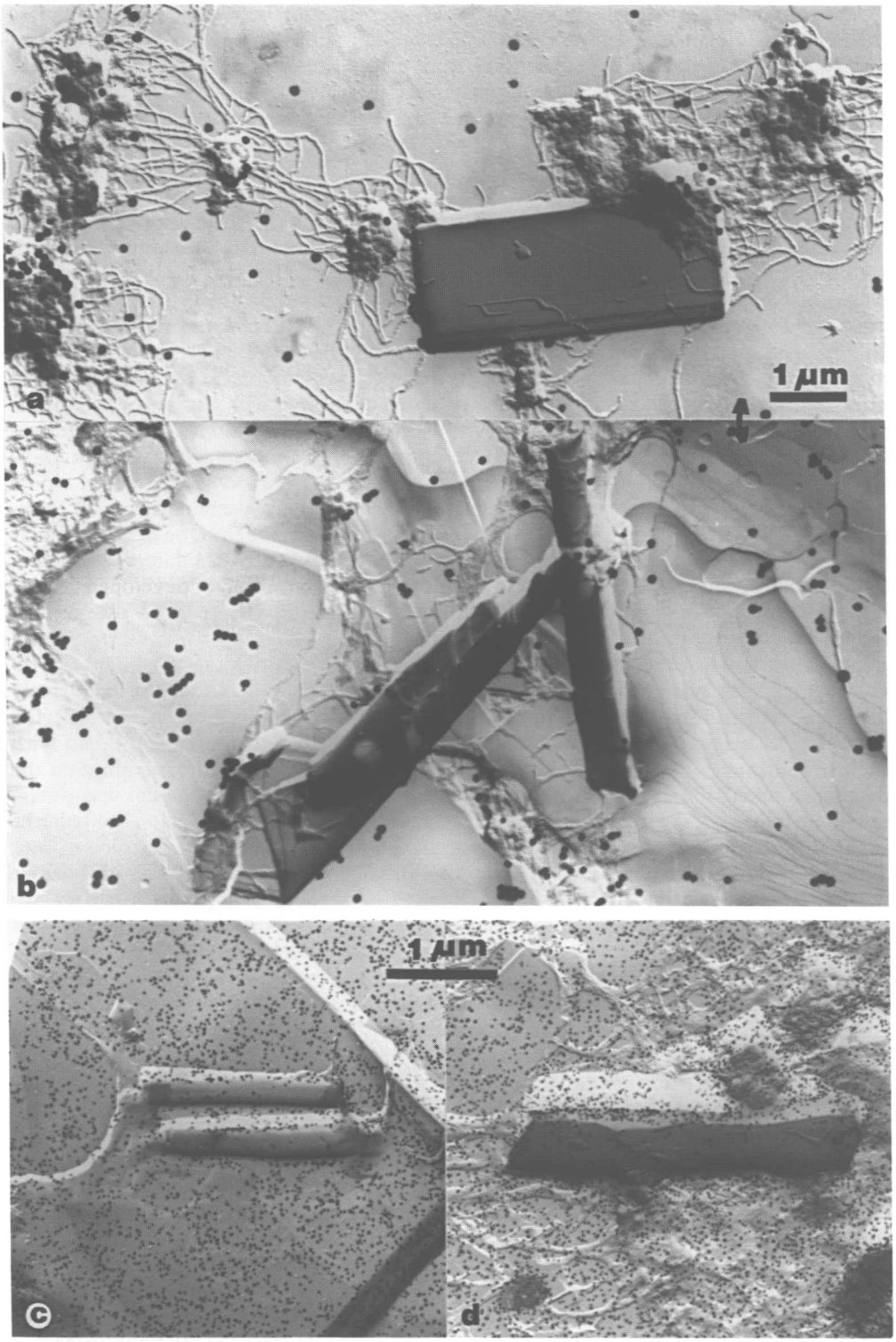
1 µm
a
b
1 µm
c
d

his laboratory facilities in order to prepare the labelled compound. This work was financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 'Vectorial Membrane Processes' 312).

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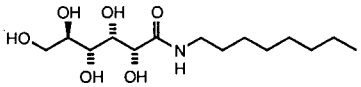
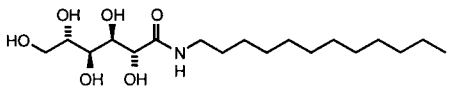
Fig. 4. Electron micrographs of autoradiographs representing ultrastructures formed by an equimolar mixture of the tritium-labelled *N*-octyl-[6-^{3}H]-D-gluconamide (D-[6-^{3}H]-Glu-8) and unlabelled *N*-dodecyl-L-mannonamide (L-Man-12). Pt/C shadowed. Emulsion L4 (Ilford). The samples were taken at stage 3 gelled solution. (a) and (b) Unlabelled anisotropic sheets (L-Man-12) surrounded by labelled helical fibres and isotropic sheets (D-[6-^{3}H]-Glu-8). Development after 83 days in Phenidon developer (Lettre & Paweletz, 1966). (c) and (d) Unlabelled rolled-up sheets and labelled helical fibres or isotropic sheets, developed after 63 days in GEA-developer (Wisse & Tates, 1968).

APPENDIX

Chemical structures of *N*-octyl-D gluconamide (D-Glu-8) and *N*-dodecyl-L-mannonamide (L-Man-12):



N-octyl-D-gluconamide (D-Glu-8)



N-dodecyl-L-mannonamide (L-Man-12)